



## Field

The invention provides a new distinct somaclone of rose scented geranium *P. graveolens* christened as "Parimal" characterized by distinct morphology and improved oil yield determining parameters. The said novel somaclone has been developed from callus through *in vitro* tissue culture technique. The plant possesses characteristic leaf morphology, vigorous growth, improved oil content per plant, herb yield and oil yield. The oil produced by this somaclone resembles the oil produced by the commercial type of bourbon geranium oil in that has equivalent ratio of citronellol and geraniol.

## Background of the Invention

The present invention relates to a new and distinct somaclone of rose scented geranium *Pelargonium graveolens*, a member of the family Geraniaceae, which is a stable somaclone of the Indian cultivar Hemanti. The oil of geranium finds extensive use in perfumery, cosmetics, food and pharmaceutical industries (Narayana, 1986). Geranium was introduced in India in the beginning of this century and since then its cultivation and production of oil remained restricted to the high altitude regions of Ootacamund, Kodaikanal and Yercaud areas in South India (Narayana, 1986). India produces hardly 5 tonnes of geranium oil annually as against its own requirement of approximate 100 tonnes per year (Narayana 1986). The production of oil has declined gradually over the years and much of the requirement is met by imports. This may be due to narrow genetic base, resulting partially from sexual infertility, vegetative mode of propagation and restricted area of cultivation.

In India, two cultivars of geranium are mainly known; these are the types that produce Algerian or Tunisian quality and the Bourbon or the Reunion quality of geranium oils; these are given the names *Pelargonium x graveolens* cv Hemanti and *Pelargonium x graveolens* cv Bipuli, respectively. The cultivar Hemanti was found to be more adaptive than the cultivar Bipuli both in the hilly regions as well as in the unconventional areas of the plains. On the other hand, the cultivar Bipuli scored better than the Hemanti mainly because of its better oil yield and improved organoleptic value of the oil. The perfumery value of geranium oil is mainly determined by the ratio of citronellol and geraniol and almost equal contents of these two major terpenoid components is generally considered to be the characteristic of the best quality geranium oil.

In an attempt to extend the area of cultivation of geranium to the Indo-gangetic plains, the better adaptive Hemanti cultivar was introduced in the Lucknow conditions in India. Subsequently, efforts were made through different biotechnological methods to develop clones with high content and desirable composition of the oil. The calliclone "Parimal" evolved through this invention represents improvement over the wild type Hemanti parental cultivar both in terms of quality and quantity determining traits of the essential oil. This particular somaclone, being consistently stable over three years of field trials under Lucknow conditions, can offer an improved cultivar for commercial exploitation.

### **Objects of the invention**

The main objective of the invention is to develop useful somaclonal variants of rose scented geranium christened as "Parimal" which are distinct, stable and uniform through successive generations.

Another object is to develop somaclonal variants yielding high quality and quantity of essential oil.

### **Summary of the invention:**

Accordingly the present invention provides a novel variety of rose scented geranium called "Parimal", said plant developed through *in vitro* tissue culture methods from callus cultures. The invention further provides a useful somaclonal variant "Parimal" of rose scented geranium (*Pelargonium graveolens* cv. Hemanti) yielding high quality and quantity of essential oil.

### **Detailed description:**

In accordance with the above and other objects, the invention provides a novel somaclone of rose scented geranium called "Parimal". The said plant has been developed though *in vitro* tissue culture methods from callus cultures initiated from stem explants and cultured on modified Murashige and Skoog's medium (MS), 1962.

The invention further provides a useful somaclonal variant "Parimal" of rose scented geranium (*Pelargonium graveolens* cv. Hemanti) having the following morphological characteristics.

Plant height	85-95 cm
Growth habit	spreading, round canopy
Stem	green, internodes 8-9 cms long, nodes pinkish
Branches	91-101
Leaf	
number per branch	10-25
texture	pubescent
shape	thick, serrated with round lobes
number of primary lobes	3
secondary lobes	12-15
Tip	Blunt
Leaf area	20-28 cm <sup>2</sup>
Petiole	~8 cm (pinkish)
Morphological marker	Less dentated leaf morphotype (Ldl)
Leaf / stem ratio (L/S)	2.1
Herb yield/ plant	4.5 kgs.
% oil content	0.18 (mean)
Oil yield /plant	7.1 gms. (mean)
Oil yield (kg/ha)	71.1 (mean)

This invention further provides the useful somaclonal variant "Parimal" of rose scented geranium (*P. graveolens* cv. Hemanti) having the following essential oil quality determining characteristics. The average and mean percentage of the respective constituents of the essential oil are provided hereinbelow:

Essential oil constituents	Percentage (%)
Linalool	9.1
Cis + Trans rose oxide	0.60
Menthone	0.2
Isomenthone	7.8
Citronellol	27.6
Geraniol	22.6
Citronellyl formate	5.9

Geranyl formate	2.1
Citronellyl acetate	0.2
$\beta$ -caryophyllene	0.2
6,9 guaiadiene	0.6
citronellyl butyrate	1.0
10 Epi- $\gamma$ -eudesmo	7.3
Citronellyl tiglate	0.8

This invention further provides the said somaclone "Parimal" which exhibited uniqueness with respect to the following RAPD primers: MAP-05, MAP-12, MAP-15 and MAP-16.

The methods and examples for the development of the said novel plant "Parimal" are provided herein below, for illustration of the invention and should not be construed as limitations on the concept of the invention. Modifications that may be apparent to those skilled in the art are deemed to fall within the scope of the present invention.

#### **Brief description of the accompanying drawings:**

The invention is further illustrated with reference to the following drawings wherein:

**Figure 1:** Field grown plant of somaclone "Parimal" having large round canopy and profuse branching.

**Figure 2:** Field grown plant of wild type parent spreading prostrate canopy and comparatively less branching.

**Figure 3:** Leaves of somaclone "Parimal" and wild type showing comparatively less dentation in the leaf of the former than that in the latter.

**Figure 4(a-d):** Variation in the RAPD profiles of wild type parent (C) with that of the selected somaclone "Parimal" (marked with (→)arrow); a: primer MAP-5; b: primer MAP-12; c: primer MAP-15; d: primer MAP-16.

#### **Methods for development of the novel somaclonal variant "Parimal":**

Healthy robust shoots, raised by subculturing the organogenic calli on modified MS medium with concentrations of BAP and NAA, were transferred to half strength MS medium with a concentration of indole butyric acid (IBA) for rhizogenesis. The stem explants were cultured on

modified Murashige and Skoog's medium (MS), 1962 containing the following (in mg/L)-  
NH<sub>4</sub>NO<sub>3</sub> (1650), KNO<sub>3</sub> (1900), CaCl<sub>2</sub>.2H<sub>2</sub>O (400), MgSO<sub>4</sub>.7H<sub>2</sub>O (370), KH<sub>2</sub>PO<sub>4</sub> (170),  
Na<sub>2</sub>EDTA.2H<sub>2</sub>O (7.2), FeSO<sub>4</sub>.7H<sub>2</sub>O (27.8), MnSO<sub>4</sub>.4H<sub>2</sub>O (22.3), ZnSO<sub>4</sub>.7H<sub>2</sub>O (8.6), H<sub>3</sub>BO<sub>3</sub>  
(6.2), KI (0.83), Na<sub>2</sub>MoO<sub>4</sub>.2H<sub>2</sub>O (0.25), CuSO<sub>4</sub>.5H<sub>2</sub>O (0.025), CoCl<sub>2</sub>.6H<sub>2</sub>O (0.025), Glycine  
(2.0), Nicotinic acid (0.5), Pyridoxine HCl (0.5) and Thiamine HCl (0.1) with proportion of  
naphthelene acetic acid (NAA) and Kinetin (Kn)[2:1]. The best organogenesis (85%) could be  
achieved by substituting Kinetin with 6-Benzylaminopurine (BAP).

The rooted plants were initially hardened under glass house conditions and were subsequently transferred to the field in the month of October 1996, in replicated trials. Based on the superior performance for vigorous growth resulting from extensive branching and enlarged canopy size, comparatively better oil yield over that of the control wild type cultivar and distinctively improved oil quality in terms of the major terpenoid constituents, the plant of this invention (Somaclone "Parimal") was selected for further observation and evaluations. During the monsoon seasons of 1996-97, this selected somaclone along with control parent were maintained under glass-house conditions through stem-cuttings which also maintained clonal purity. The selected clone "Parimal" was grown in field in replicated trials along with its parent Hemanti cultivar for two consecutive winter-summer seasons of 1997-98 and 1998-99, while during the intermediate monsoon season, they were maintained under glass-house conditions. The clone "Parimal" maintained its vigorous growth through extensive branching, better oil content and improved oil quality over the three consecutive years. The characteristics of this particular clone (in terms essential oil quantity and quality determining traits) in relation to that of the control parent and other clones have been represented in Tables-2 and 3. The results clearly demonstrate the outstanding performance of clone "Parimal" over that of the control wild type parent and other selected clones under Lucknow field conditions, indicating the feasibility of its commercial cultivation in the indo-gangetic plains. It is pertinent to mention here that the oil profile of somaclone "Parimal" is superior than that of the wild type parent in having equal content of citronellol and geraniol which is a critical characteristic of best quality commercial geranium oil. The field grown plant of "Parimal" having large round canopy is shown in Fig.1 and Fig.2 depicts the wild type parent. The leaves of "Parimal" are as shown in Fig.3.

## Evidences of uniformity and stability

The somaclone "Parimal" has remained stable and uniform for its morphological characters and showed consistency in performance for various oil yield and quality determining attributes during its field evaluation under Lucknow conditions over three consecutive years of 1996-97, 1997-98 and 1998-99 are depicted in (Tables 1-3).

### Morphological description of the somaclone "Parimal" and its wild type parent

- 1) Genus - *Pelargonium*
- 2) Species - *graveolens*
- 3) Family - Geraniaceae
- 4) Common name - rose-scented geranium

**Table 1 :** Comparative analysis of oil yield and quality determining traits in the field grown morphologically distinct somaclones, "Parimal" and wild type *Pelargonium graveolens* CV. Hemanti (control).

Somaclone design-ation	Morpho-types	Oil yield/plant (g)	% contents of major terpenoids in the essential oil of geranium								
			Linal-ool	Cis+trans rose oxide	Isomen-thone	Citron-ellol	Geran-iol	Citronel-lyl for-mate	Geranyl formate	6,9-guai-adiene	10-epi-□-eude-smol
A	Highly dentated leaf	4.5±0.5	1.1±0.2	1.6±0.4	9.6±1.8	49.8±1.4	2.0±0.5	13.0±0.4	0.5±0.3	1.3±0.6	2.9±0.5
B		3.3±0.7	1.1±0.2	1.3±0.3	9.0±2.5	52.6±1.9	2.3±0.6	11.7±1.3	0.3±0.15	1.3±0.4	3.0±0.7
C		8.6±3.7	0.9±0.1	1.2±0.2	10.3±1.3	51.7±1.1	1.8±0.4	11.1±0.9	0.23±0.03	1.3±0.4	2.9±0.5
D		4.6±1.2	1.0±0.3	0.9±0.3	10.6±2.1	48.6±4.5	2.3±1.1	10.2±2.7	0.6±0.45	1.2±0.4	4.4±1.4
Parimal	Less dentated leaf	11.3±2.8	9.1±1.6	0.6±0.12	7.8±0.53	27.6±2.1	22.6±3.2	5.9±0.2	2.1±0.4	0.6±0.12	7.3±0.95
L	Less dentated leaf	8.0±4.0	8.5±2.8	0.6±0.1	7.7±0.5	28.7±4.3	22.3±2.8	5.6±0.7	2.0±0.2	0.7±0.1	7.4±1.1
M		4.5±0.8	7.8±1.5	0.7±0.2	6.9±1.5	29.2±3.9	20.4±2.1	4.8±0.7	1.8±0.2	0.7±0.03	8.6±1.8
N		5.1±0.6	7.5±1.3	0.6±0.2	7.1±1.2	29.1±4.4	19.5±3.1	4.7±0.8	1.8±0.6	0.6±0.05	8.2±1.6
W (control)	Highly dentated	3.8±0.33	0.7±0.1	1.5±0.3	9.5±0.1	53.1±0.7	1.6±0.14	12.1±0.6	0.23±0.03	2.0±0.2	3.2±0.2

**Table 2 : Morphological characteristics of somaclone "Parimal" and its wild type parent**

Morphological parameters	Parimal	Wild type
Plant height	85-95 cm	35-45 cm
Growth habit	spreading, round canopy	Spreading prostrate
Stem	green, internodes 8-9 cms long, nodes pinkish	Greenish magenta purplish at the nodes, 5-10 mm in diameter, densely hairy
Branches	91-101	10-45
Leaf		
Number per branch	10-25	25-30
Texture	pubescent	pubescent
Shape	thick, serrated with round lobes	thick hairy highly serrated
Number of primary lobes		3 to 5
secondary lobes	3 12-15	15-30
Tip	Blunt	Blunt
Leaf area	20-28 cm <sup>2</sup>	30-36 cm <sup>2</sup>
Petiole	~8 cm (pinkish)	~5 cm (green)

**Table -3:** Oil yield and quality determining traits of somaclone "Parimal" and its wild type parent.

Trait	Somaclone "Parimal"	Wild type
1. Morphological marker	Less dentated leaf morphotype (Ldl)	Highly dentated leaf morphotype (Hdl)
2. Leaf / stem ratio (L/S)	2.1	1.5
3. Herb yield/ plant	4.5 kgs.	1.9 kgs
4. % oil content	0.18	0.15
5. Oil yield /plant	7.1 gms.	2.5 gms.
6. Oil yield (kg/ha)	71.1	25.1
7. Oil quality		
i) Linalool	9.1	0.7
ii) <i>Cis</i> + <i>Trans</i> rose oxide	0.60	1.5
iii) Menthone	0.2	0.3
iv) Isomenthone	7.8	9.5
v) Citronellol	27.6	53.1
vi) Geraniol	22.6	1.6
vii) Citronellyl formate	5.9	12.1
viii) Geranyl formate	2.1	0.2
ix) Citronellyl acetate	0.2	0.9
x) $\beta$ -caryophyllene	0.2	1.5
xi) 6,9 guaiadiene	0.6	2.0
xii) citronellyl butyrate	1.0	2.2
xiii) 10 Epi- $\gamma$ -eudesmol	7.3	3.2
xiv) Citronellyl tiglate	0.8	1.6



### Type and frequency of variants during multiplication and maintenance of the somaclone

No variants has been recovered during multiplication, maintenance and testing of the somaclone "Parimal" in experimental plots, screened carefully in 1996-97, 1997-98 and 1998-99 winter-summer cropping seasons. The three year's observations on somaclone "Parimal" were statistically analysed for significance of differences in the morphological oil yield determining attributes and the concentrations of 9 geranium oil quality determining terpenoid constituents. The intraclonal variation in clone "Parimal" was found statistically insignificant over three year's field trial.

### Statement of distinction

The somaclones "Parimal" are distinct in having large round canopy, less dentated round leaf morphology, extensive branching and absence of flowering (Table 1). Its oil profile is distinct from the wild type parent cultivar in having higher proportion of geraniol and lower proportion of citronellol, lower content of cis + trans rose oxide and citronellyl formate, 6,9-guadiene, higher content of linalool, geranyl formate and 10-epi-y endesmol (Table-2). Its RAPD profile was different from the wild type parent in certain aspects while had certain features common in both.

### Characterization through RAPD markers

Using 13 random primers (MAP 01 to MAP 16) with following sequence description a comparison was made for somaclone "Parimal" and its wild type parent to establish the relatedness between them.

Primer	Base sequence
MAP01	GTGCAATCAG
MAP02	AGGATACGTG
MAP04	AAGATAGCGG
MAP05	GGATCTGAAC
MAP06	TTGTCTCAGG
MAP07	GTCCTACTCG
MAP09	TGCGCGATCG
MAP10	AACGTACGCG
MAP11	GCACGCCGGA

Primer	Base sequence
MAP12	CACCCTGCGC
MAP14	GGACTCCACG
MAP15	AAGATAGCGG
MAP16	CTATCGCCGC

This analysis could narrow down with usage of six MAP primers (MAP 04, MAP 05, MAP 10, MAP 14, MAP 15, MAP 16) which in turn were utilized for genotypic pair analysis in order to assess genetic / molecular distances. The following table 4 gives the similarity index between the two genotypes in paired test.

Table 4 : Similarity percentage for amplified bands (paired analysis) of somaclone "Parimal" and wild type parent

Primer	Genotypic pair (somaclone "Parimal"/ wild type)
MAP 04	93
MAP 05	80
MAP 10	60
MAP 14	90
MAP 15	83
MAP 16	66

It is evident from similarity indices (paired test) that somaclone "Parimal" and wild type parent are genetically distinguishable (Table 4). Further, the primer MAP 10, MAP 14 showed least similarity while primers MAP 04 and MAP15 showed close relatedness. The primers MAP05 and MAP16 showed intermediate degree of similarity. These observations on percent similarity confirm the relatedness of somaclone "Parimal" with the wild type parent, at the same time highlight the distinctness of the former at the genetic level. The complete RAPD profile for PCR amplified DNA segments using 12 primers is available providing evidence of distinctiveness of the clone with respect to the control parent as shown in Figure 4.

### Advantages:

1. "Parimal" is well adaptive to the unconventional area of cultivation i.e. northern Indian plains.
2. "Parimal" represents improvement over its wild type parental cv. "Hemanti" with respect to the following quantitative traits:
  - (a) Larger and denser canopy;
  - (b) Greater herb yield;
  - (c) Higher leaf : stem ratio;
  - (d) Higher oil content; and
  - (e) Much higher oil yield (both on per plant and per hectare basis).
3. "Parimal" represent improvement over its wild type parental cv. "Hemanti" with respect to following major essential oil quality determining traits:
  - (a) About equal citronellol:geraniol ratio;
  - (b) Higher rhodinol content;
  - (c) Higher linalool concentration;
  - (d) Lower concentration of 6,9-guaiadiene.
4. The somaclone "Parimal" showed consistency in performance for all the above mentioned oil yield and quality determining attributes during its field evaluation under Lucknow conditions over three consecutive years.
5. "Parimal" showed distinctiveness even at genetic level with respect to RAPD primers, MAP-05, MAP-12, MAP-15 and MAP-16.